

## SPECIFICATION

TITLE OF THE INVENTION ;

OLIGOSACCHARIDE SYNTHESIZER

5 DESCRIPTION OF THE INVENTION:

FIELD OF THE INVENTION:

The present invention relates to a technique for  
oligosaccharide synthesis and separation processing,  
particularly to a oligosaccharide synthesizer for  
10 automating these types of processing.

BACKGROUND OF THE INVENTION:

A complex carbohydrate in a cell plays an  
important role in information transfer, identification  
15 among cells and recognition of a virus, cancer cell,  
blood type and others. Clarification of  
oligosaccharide functions is ranked as one of the  
major tasks that come after the study of genome. An  
oligonucleic acid and peptide synthesis method have  
20 been already established and automated.

Oligosaccharide synthesis, however, has many problems  
yet to be solved.

In an effort to clarify the functions of  
oligosaccharide, establishment of oligosaccharide  
25 synthesis method and creation of an effective

synthesizer have been long awaited. At present, the following three methods of oligosaccharide synthesis are practiced:

(1) Chemical synthesis

5       (2) Genetically recombinant cell or fermentation  
by microorganism

(3) Synthesis by glycosyltransferase

The method (1) is characterized by complicated steps of synthesis, since sequential synthesis of  
10 target oligosaccharides is performed while protecting  
the OH group other than the OH group for chemical  
bondage. The method (2) is characterized in that,  
although a great numbers of target oligosaccharides  
can be obtained, subsequent steps of purification are  
15 complicated. The method (3) was developed to solve the  
problems encountered in the complicated steps in  
methods (1) and (2). It is disclosed in the Japanese  
Patent Laid-Open Publication No. 11-42096 (1999), for  
example. The method (3) is based on synthesis by  
20 selective glycosyltransferase, and does not require  
protection of the OH-group, as in method (1). Further,  
there is not much quantity of by-products, and  
purification process subsequent to synthesis is easy.

A oligosaccharide synthesizer is disclosed in the  
25 Japanese Patent Laid-Open No. 05-500905 (1993).

When an actual apparatus is used to carry out the synthesis of a oligosaccharide based on the aforementioned method (3) a batch system is used at present, wherein separation and purification of products are carried out for each step in the reaction of a plurality of sugars performed one after another. The next reaction is carried out after that. Manpower is essential to complete the entire processing, according to this method.

10 In the apparatus disclosed in the aforementioned Japanese Patent Laid-Open No. 05-500905, according to the order of sugars to be reacted, reaction columns and separation/purification means must be connected on a continuous basis.

15 To put it another way, even if the same sugars are reacted, the same number of reaction columns and separation/purification means as that of sugars are required. This requires a large-scale system of apparatuses.

20 Further, glycosyltransferase needed for reaction is generally very expensive. Any arrangement for permitting repeated use of glycosyltransferase is not taken into account.

25 SUMMARY OF THE INVENTION:

The object of the present invention is to provide a oligosaccharide synthesizer that ensures effective synthesis of a plurality of sugars, and recovery and reuse of glycosyltransferase.

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#### BRIEF DESCRIPTION OF DRAWINGS:

Fig. 1 is a system configuration diagram and flow diagram according to Embodiment 1;

Fig. 2 is a system configuration diagram and flow diagram according to Embodiment 2; and

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Fig. 3 is a system configuration diagram and flow diagram according to Embodiment 3.

#### DESCRIPTION OF THE INVENTION:

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The present invention for achieving the aforementioned object is characterized in that, after various sugar nucleotide solutions and glycosyltransferases (or primers) have been mixed, they are introduced into a reaction tank where primers (or glycosyltransferases) are immobilized, whereby sugars are chemically bonded with primers one after another. After that, eluate is led into the ultrafiltration column from the reaction tank.

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The present invention is also characterized by comprising a flow path for ensuring that the

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glycosyltransferase or primer separated by the ultrafiltration column is returned into a container for storing each solution in a sample injector.

This method permits effective synthesis of  
5 oligosaccharides. It also allows recovery of high-priced glycosyltransferase for reuse.

The present invention permits continuous and automatic synthesis of complicated oligosaccharides..

Further, it allows glycosyltransferases to be  
10 recovered for reuse.

#### Detailed Description of the Preferred Embodiments:

##### Embodiment 1

Fig. 1 is a system configuration diagram according  
15 to Embodiment 1.

A oligosaccharide synthesizer comprises:

a buffer 1 best suited to reaction;

a pump 2 for feeding the buffer;

a mixing tank 4 for cooling and storing sugar  
20 nucleotides (X-Sn) 10 through 13 and glycosyltransferases (En) 14 through 17 and for mixing them after weighing;

a sample injector 3 for injecting the mixture into a flow path;

25 a reaction tank 5 for storing an immobilized

primer (P);

an ultrafiltration column 6 for separating glycosyltransferase from unreacted sugar nucleotide (X-Sn) and nucleotide (X) as a product of reaction;

5 a six-way valve 7 for switching to determine whether the solution leaching out of the ultrafiltration column 6 should be discharged into the drain or an after-stage flow path switch valve 8;

a flow path switch valve 8 for leading the  
10 solution into the drain or each of the contains of glycosyltransferases (En) 14 through 17 of the sample injector 3; and

a controller 9 for controlling these components.

The aforementioned ultrafiltration column 6  
15 incorporates an ultra-filter and separates glycosyltransferase from unreacted sugar nucleotides (X-Sn) and nucleotides (X) as products of reaction, according to molecule size. Normally, the molecular weight of glycosyltransferase is of the order of tens  
20 of thousands, and those of the sugar nucleotides (X-Sn) and nucleotides (X) as products of reaction are of the order of several hundreds. This makes separation easy. The isolated glycosyltransferase is fed to the side "a" of the bottom of the ultrafiltration column 6,  
25 whereas the sugar nucleotides (X-Sn) and nucleotides

(X) are fed to the side "b" on the lateral face of the ultrafiltration column 6.

Further, a black square in the six-way valve 7 indicates a sealed port. Accordingly, as shown in Fig. 1, when the portion indicated by the solid line forms a flow path, the solution running out of the side "a" of the ultrafiltration column 6 is stopped at the sealed position of the six-way valve 7.

In the present embodiment, the glycosyltransferase (En) refers to galactosyltransferase, N-acetyl glucosaminyltransferase, N-acetyl galactosaminyltransferase, fucosyltransferase, sialyltransferase, mannosyltransferase, etc.

Further, sugar nucleotide solution (X-Sn) refers to uridine-5'-diphosphogalactose, uridine-5'-diphospho-N-acetylglucosamine, uridine-5'-diphospho-N-acetylgalactosamine, guanosine-5'-diphosphofucose, guanosine-5'-diphosphomannose, cytidine-5'-monophospho-N-acetylneuraminic, etc.

Primer (P) denotes a water soluble polymer, and refers to a biopolymer such as protein, glycoprotein, glycopeptide, lipid, glycolipid, oligosaccharide and polysaccharide, as well as synthetic polymer such as polyacrylamide derivative described in the aforementioned Japanese Patent Laid-Open Publication

No. 11-42096.

(Further, the primer having chemically bonded with sugar ( $S_n$ ) hereinafter referred to as "primer (P- $S_n$ )").

The primer (P) immobilized on a certain carrier of solid state is an immobilized primer (P). A thermostatic bath incorporating this immobilized primer (P) is a reaction tank 5. In the present embodiment, the primer (P) in the reaction tank 5 is bonded with sugar ( $S_0$ ) in advance.

The following describes the operation of this apparatus with reference to Fig. 1:

Here primer P and sugars  $S_1$ ,  $S_2$  and  $S_3$  are assumed to have been synthesized in the order of P- $S_1$ - $S_2$ - $S_3$ . In practice, however, there is no restriction to the order of sugars  $S_1$ ,  $S_2$  and  $S_3$ .

The difference in the synthesis of sugars  $S_1$ ,  $S_2$  and  $S_3$  is found in that they are assigned with the reaction temperature and reaction time best suited to each of them. Accordingly, when reaction is to be made in the order or P- $S_1$ - $S_2$ - $S_3$ , the reaction temperature and reaction time in the reaction tank 5 are set in conformity to each sugar, and the following five steps are repeated three times in principle.

Further, when the optimum buffer is required in the synthesis of each, a plurality of buffers 1 are



provided and a pump equipped with a low-pressure gradient function and capable of selecting a buffer is employed. This will easily meet the requirements.

Step 1:

5 Buffer 1 is fed by a pump 2 at a predetermined flow rate to wash the flow path. The six-way valve 7 and flow path switch valve 8 are set to the drain position to initialize the apparatus. In this case, the interior of the sample injector 3 is washed and  
10 the temperature of the reaction tank 5 is also set.

Step 2:

In the sample injector 3, a certain amount of sugar nucleotide (X-S1) 10 and its glycosyltransferase (E1) 14 are weighed and are mixed in the mixing tank 4.  
15 After that, the mixture is injected into the flow path.

Step 3:

The aforementioned mixture is introduced into the reaction tank 5 where primer (P) is immobilized, and is left to reaction at a certain temperature for a  
20 certain period of time. During reaction time, the flow rate of the pump 2 is constant or zero. When setting the flow rate of the pump, it is necessary to make sure that the sugar nucleotide (X-S1) 10 and its glycosyltransferase (E1) 14 do not flow out of the  
25 reaction tank 5 during the reaction time.

Step 4:

After expiration of reaction time, the solution coming out of the reaction tank 5 is led into the ultrafiltration column 6. It is fed for a certain time period, and is separated by an ultrafilter membrane. The solution coming out of the reaction tank 5 includes the glycosyltransferase (E1) 14, unreacted sugar nucleotide (X-S1) 10 and nucleotides (X) as products of reaction.

10 In this case, the six-way valve 7 functions as a flow path indicated by a solid line in Fig. 1. The filtered unreacted sugar nucleotide (X-S1) 10 and nucleotides (X) as products of reaction flow into the drain, and the glycosyltransferase (E1) 14 remains in  
15 the ultrafiltration column 6 since the flow path of the six-way valve 7 is sealed.

Step 5:

After separation, the six-way valve 7 is switched over to the flow path indicated by a dotted line in  
20 Fig. 1, and the glycosyltransferase (E1) 14 remaining in the ultrafiltration column 6 is fed to the side of the flow path switch valve 8. The flow path switch valve 8 is switched over to the flow path connected to a bottle for the glycosyltransferase (E1) 14. Then the  
25 glycosyltransferase (E1) 14 is captured and stored.

The aforementioned steps are repeated by replacing the sugar nucleotide (X-Sn) and glycosyltransferase (En), whereby the introduced sugars (Sn) are bonded to the primer (P) in the reaction tank 5 one after another, and a oligosaccharide is formed by synthesis.

To release the synthesized oligosaccharide from the solid state carrier upon termination of all reactions, an enzyme for this purpose is required. The enzyme for release is led from the sample injector 3, and is made to react in the reaction tank 5 for a certain time. After that, it is separated from the enzyme for release by the ultrafiltration column 6, and synthesized oligosaccharide ( $S_0-S_1-S_2-S_3$ ) is captured on the drain side of the six-way valve 7.

The  $S_0$  denotes the sugar first attached to the primer (P).

According to the present embodiment, efficient synthesis of a oligosaccharide can be carried out and the glycosyltransferases used for synthesis can be recovered for reuse.

#### [Embodiment 2]

Fig. 2 is a system configuration diagram of the Embodiment 2.

The oligosaccharide synthesizer contains:  
a buffer 1 best suited to reaction;

a pump 2 for feeding the buffer;

a mixing tank 4 for cooling and storing sugar  
nucleotides (X-Sn) 10 through 13 and  
glycosyltransferases (En) 14 through 17 and for mixing  
5 them after weighing;

a sample injector 3 for injecting the mixture into  
a flow path;

a reaction tank 5 for storing an immobilized  
primer (P),

10 a recycling six-way valve 18 for returning the  
solution coming out of the reaction tank 5, to the  
inlet of the pump 2;

a trap bottle 19 enclosed to once trap the  
solution coming out of the reaction tank 5, at the  
15 position just before the pump 2;

an ultrafiltration column 6 for separating  
glycosyltransferase from unreacted sugar nucleotide  
(X-Sn) and nucleotide (X) as a product of reaction;

a six-way valve 7 attached thereto;

20 a flow path switch valve 8 for recovering  
glycosyltransferase after ultrafiltration; and

a controller 9 for controlling these components.

In the present embodiment, the primer (P) in the  
reaction tank 5 is bonded with the sugar (S<sub>0</sub>) in  
25 advance. Further, a black square in the six-way valve

18 indicates a sealed port, as in the case of six-way valve 7.

The following describes the operation of this apparatus with reference to Fig. 2:

5        Here primer P and sugars  $S_1$ ,  $S_2$  and  $S_3$  are assumed to be reacted in the order of P- $S_1$ - $S_2$ - $S_3$ . In practice, however, there is no restriction to the order of sugars  $S_1$ ,  $S_2$  and  $S_3$ .

10        When synthesis is made in the order of P- $S_1$ - $S_2$ - $S_3$ , the reaction temperature and reaction time in the reaction tank 5 are set in conformity to each sugar, and the following five steps are repeated three times in principle:

Step 1:

15        Buffer 1 is fed by a pump 2 at a predetermined flow rate to wash the flow path. The six-way valve 7 and flow path switch valve 8 are set to the drain position, and the recycling six-way valve 18 initializes the apparatus, using the flow path  
20        indicated by a solid line. In this case, the interior of the sample injector 3 is washed and the temperature of the reaction tank 5 is also set.

Step 2:

25        A certain amount of sugar nucleotide (X- $S_1$ ) 10 and its glycosyltransferase are weighed and are mixed in

the mixing tank 4. After that, the mixture is injected into the flow path by the sample injector 3.

Step 3:

The aforementioned mixture is introduced into the reaction tank 5 where primer (P) is immobilized, and is left to reaction at a certain temperature for a certain period of time. During reaction time, the flow rate of the pump 2 is constant. The recycling six-way valve 18 is switched to form a flow path indicated by a dotted line.

Accordingly, the injected sugar nucleotide (X-S1) 10 and its glycosyltransferase (E1) 14 pass through the reaction tank 5 repeatedly during the reaction time.

Step 4:

After expiration of reaction time, the recycling six-way valve 18 is reset to the flow path indicated by a solid line. Then the solution coming out of the reaction tank 5 is led into the ultrafiltration column 6. It is fed for a certain time period, and is separated by an ultrafilter membrane. The solution coming out of the reaction tank 5 includes the glycosyltransferase (E1) 14, unreacted sugar nucleotide (X-S1) 10 and nucleotides (X) as products of reaction.

In this case, the six-way valve 7 functions as a flow path indicated by a solid line in Fig. 1. The filtered unreacted sugar nucleotide (X-S<sub>1</sub>) 10 and nucleotides (X) as products of reaction flow into the drain, and the glycosyltransferase (E<sub>1</sub>) 14 remains in the ultrafiltration column 6 since the flow path of the six-way valve 7 is sealed.

Step 5:

After separation, the six-way valve 7 is switched over to the flow path indicated by a dotted line in Fig. 2, and the glycosyltransferase (E<sub>1</sub>) remaining in the ultrafiltration column 6 is fed to the side of the flow path switch valve 8. The flow path switch valve 8 is switched over to the flow path connected to a bottle for the glycosyltransferase (E<sub>1</sub>) 14. Then the glycosyltransferase (E<sub>1</sub>) 14 is captured and stored.

The synthesized oligosaccharide (P-S<sub>1</sub>-S<sub>2</sub>-S<sub>3</sub>) is separated from the solid state carrier upon termination of all reactions, and is captured in the same manner as in Embodiment 1.

The major difference from the Embodiment 1 is found in the Step 3. In the present embodiment, the injected sugar nucleotide and glycosyltransferase are fed into the reaction tank 5 repeatedly at a certain flow rate, whereby permitting effective agitation and

promoting reactions. Further, what should be taken care of in the aforementioned Embodiment 1, i.e. the relationship between reaction time and flow rate, need not be taken in account, with the result that easy operation is ensured.

[Embodiment 3]

Fig. 3 is a system configuration diagram representing the present embodiment.

The oligosaccharide synthesizer comprises:

- 10 a buffer 1 best suited to reaction;
- a pump 2 for feeding the buffer;
- a mixing tank 4 for cooling and storing sugar nucleotides (X-Sn) 10 through 13 and for mixing them with the primer (P) 25 bonded with sugar ( $S_0$ ) in advance after weighing;
- 15 a sample injector 3 for injecting the mixture into a flow path;
- reaction columns 20 through 23 having a temperature regulating function for immobilizing glycosyltransferases;
- 20 a flow path switch valve 8 for selecting a flow path leading to the reaction column;
- a manifold 24;
- an ultrafiltration column 6 for separating primer
- 25 (P) and primer (P-Sn) from unreacted sugar nucleotide



(X-Sn) and nucleotide (X) as a product of reaction;

a six-way valve 7 attached thereto; and

a controller 9 for controlling these components.

In the present embodiment as well, the primer (P)  
5 25 in the reaction tank 5 is bonded with the sugar (S<sub>0</sub>)  
in advance.

The difference from the Embodiments 1 and 2 is  
that primer (P) is immobilized to the reaction tank 5  
in Embodiments 1 and 2, whereas glycosyltransferases  
10 are immobilized in the present embodiment. Further,  
glycosyltransferases are recovered in Embodiments 1  
and 2, while primer (P) is recovered in the present  
embodiment. It should be noted that several types of  
glycosyltransferases are used in conformity to the  
15 type of the sugar to be synthesized. This requires use  
of a plurality of reaction columns where  
glycosyltransferases are immobilized.

In the present embodiment, the aforementioned  
ultrafiltration column 6 separates the primer (P) from  
20 unreacted sugar nucleotide (X-Sn) and nucleotide (X)  
as a product of reaction according to molecular size  
by an ultrafilter membrane. The separated primer (P)  
and primer (P-Sn) are fed to the side "a" of the  
ultrafiltration column 6, and the sugar nucleotide (X-  
25 Sn) and nucleotide (X) are fed to the side "b" of the

ultrafiltration column 6.

The following describes the operation of this apparatus with reference to Fig. 3:

Here primer P and sugars  $S_1$ ,  $S_2$  and  $S_3$  are assumed  
5 to be synthesized in the order of P- $S_1$ - $S_2$ - $S_3$ . Further,  
the glycosyltransferases for catalyzing the transfer  
reaction of sugars  $S_1$ ,  $S_2$  and  $S_3$  are assumed to be  
immobilized in the reaction columns 20 through 23. In  
practice, however, there is no restriction to the  
10 order of sugars  $S_1$ ,  $S_2$  and  $S_3$ .

When synthesis is made in the order of P- $S_1$ - $S_2$ - $S_3$ ,  
the reaction temperature and reaction time in the  
reaction columns 20 through 23 are set in conformity  
to each sugar, and the following five steps are  
15 repeated three times in principle:

Step 1:

Buffer 1 is fed by a pump 2 at a predetermined  
flow rate to wash the flow path. The six-way valve 7  
initializes the apparatus, using the flow path  
20 indicated by a solid line. In this case, the interior  
of the sample injector 3 is washed. Further, the flow  
paths leading to the reaction columns 20 through 23 to  
be used are selected by the flow path switch valve 8,  
and the temperature of the reaction columns is set.

25 Step 2:

A certain amount of sugar nucleotide (X-S1) 10 and primer (P) 25 are weighed and are mixed in the mixing tank 4. After that, the mixture is injected into the flow path by the sample injector 3.

5           Step 3:

The aforementioned mixture is introduced into a reaction column where glycosyltransferase is immobilized, for example, reaction column 20, and is left to reaction at a certain temperature for a  
10           certain period of time.

          Step 4:

After expiration of reaction time, the solution coming out of the reaction column 20 is led into the ultrafiltration column 6. It is fed for a certain time  
15           period, and is separated by an ultrafilter membrane. The solution coming out of the reaction column 20 includes the primer (P-S1) formed by the primer (P) bonded with sugar (S<sub>1</sub>), and unreacted sugar nucleotide (X-S1) 10 and nucleotides (X) as products of reaction.

20           In this case, the six-way valve 7 functions as a flow path indicated by a solid line in Fig. 3. The filtered unreacted sugar nucleotide (X-S1) 10 and nucleotides (X) as products of reaction flow into the drain, and the primer (P-S1) remains in the  
25           ultrafiltration column 6 since the flow path of the

six-way valve 7 is sealed. The molecular weight of primer (P) is of the order of tens of thousands or hundreds of thousands, while those of the sugar nucleotides (X-S1) and nucleotides (X) are of the order of several hundreds. This makes separation easy.

Step 5:

After separation, the six-way valve 7 is switched over to the flow path indicated by a dotted line in Fig. 3, and the primer (P-S1) remaining in the ultrafiltration column 6 is recovered into the mixing tank 4.

When the primer (P-S1) has been recovered, it goes back to step 1, and the primer (P-S1) is mixed with the sugar nucleotide (X-S2) 11 and is fed to the reaction column. This processing is repeated until the termination of the planned sugar synthesis.

To release the synthesized oligosaccharide from the primer (P) upon termination of all reactions, an enzyme for this purpose is required. The flow path switch valve 8 is used to switch the flow path over to the reaction column (e.g. reaction column 23) where an enzyme for oligosaccharide release is immobilized. The primer (P-S<sub>1</sub>-S<sub>2</sub>-S<sub>3</sub>) as a final product led from the sample injector 3 is made to react for a certain time in the reaction column 23 and the oligosaccharide is

released from the primer. After that, it is separated from the primer by the ultrafiltration column 6 and the synthesized oligosaccharide ( $S_0-S_1-S_2-S_3$ ) is captured on the drain side of the six-way valve 7. The  
5 molecular weight of oligosaccharide ( $S_0-S_1-S_2-S_3$ ) is of the order of thousands, while that of the primer is of the order of tens of thousands or hundreds of thousands. This makes separation easy.

Here  $S_0$  denotes the sugar first attached to the  
10 primer (P).

According to the present embodiment, oligosaccharide synthesis reaction can be repeated while recovering the primer into the mixing tank 4, thereby ensuring efficient synthesis of  
15 oligosaccharides.